MYROTHECIUM SP. TRANSFORMATION AND EXPRESSION SYSTEM

Field of the invention

[0001] The present invention is related to a method to express proteins or to modify protein expression in a microbial host.

Background of the invention

[0002] Since the advent of the genetic engineering a number of living cells has been transformed with DNA in order to produce homologous or heterologous proteins or to modify their metabolism by introducing for example new metabolic pathways. The variety of cloning hosts spans from bacteria such as *Escherichia coli* till human cells and comprises organisms as diverse as yeasts, fungi or insect cells.

[0003] Among these organisms, the filamentous fungi have been the subjects of many studies. Indeed some of these organisms are industrially important, such as *Aspergillus* sp., *Trichoderma* sp. or *Fusarium* sp.

[0004] The first report of a DNA-mediated transformation of a filamentous fungi was published in 1973 using *Neurospora crassa* (Mishra, N.C. and Tatum E.L., 1973. Non-Mendelian inheritance of DNA-induced inositol independence in *Neurospora*. Proc. Nat. Acad. Sci. USA. 70:3875-3879). Later on, a protoplast transformation system has been developed for the same organism (Case *et al*, 1979. Efficient transformation of *Neurospora crassa* by utilising

hybrid plasmid DNA. Proc. Nat. Acad. Sci. USA. 76:5259-5263). Transformation of *Podospora anserina* (Stahl *et al*, 1982. Replication and expression of a bacterial-mitochondrial hybrid plasmid in the fungus *Podospora anserina*. Proc. Nat. Acad. Sci. USA. 79:3641-3645) and *Aspergillus nidulans* (Tilburn *et al*, 1983. Transformation by integration in *Aspergillus nidulans*. Gene. 26:205-221; Ballance *et al*, 1983. Transformation of *Aspergillus nidulans* by the orotidine-5'-phosphate decarboxylase gene of *Neurospora crassa*. Biochem. Biophys. Res. Comm. 112:284-289) were also reported. The same techniques have been successfully used to transform the commercially important industrial fungi *Aspergillus niger* (Buxton *et al*, 1985. Transformation of *Aspergillus niger* using the argB gene of *Aspergillus nidulans*. Gene. 37:207-214), *Aspergillus oryzae* (Gomi *et al*, 1987. Agric. Biol. Chem. 51:2549-2555) and *Trichoderma reesei* (Pentillä et al, 1988.Gene. 61:155-164).

[0005] Today transformations systems have been reported for many fungal classes (see for example the chapter on Genetic manipulation of fungi by transformation by Lemke P.A. and Peng M. in The Mycota volume II Genetics and Biotechnology, Kück ed, Springer-Verlag, 1995).

[0006] Some of these are patented (see for example patent EP0184438B1 on "Transforming *Aspergillus* and plasmids for use therein" or patent application WO9602653 on "Thermophilic fungal expression system"). Also are described parts of transformation systems such as the use of promoters or plasmids (For example EP0489718 "Process for the production of protein products in *Aspergillus oryzae* and a promoter for use in *Aspergillus*").

[0007] Enzymes are proteins that are produced by all living organisms. They are highly specific biological catalysts that speed up chemical reactions selectively.

[0008] They are produced industrially for a number

of application ranging from detergent formulation to food application (baking, fruit processing,...) (for reviews see the books of Uhlig, H., Industrial enzymes and their applications, 1998, John Wiley and sons, Inc; Enzymes in food technology, 2002, Whitehurst, R.J. and Law, B.A. eds, Sheffield Academic Press; Industrial enzymology, 1996, Godfrey, T. and West, S. eds., Macmillan Press ltd).

[0009] About 90% of all enzymes used in industrial processing are produced by fermentation of microorganisms.

[0010] Today very few species of microorganisms are used for industrial enzyme production. This is a limitation imposed by producing companies needing the widest market range for their products, including food processing.

[0011] However, this situation is changing as new opportunities outside food processing are appearing. Also the increasing introduction of productions using genetically manipulated microorganisms strengthens the need to find new organisms to be used. The genetic engineering allows also the possibility to produce enzymes naturally produced by microorganims having poor growing properties in fermentors.

[0012] Besides enzyme production, genetically modified microorganisms are used for many other applications such as but not restricted to therapeutical drugs production (antibiotics, anticancer,...), new metabolic pathway expression,...

[0013] Therefore, there is still a need for expression systems that would allow high or modulated levels of production of various compounds.

[0014] The diversity of the microbial world is today largely underexploited. It has been estimated that less than 1% of the microorganisms living on earth have already been discovered. Among those identified, very few have been

the subject of investigations other than morphological or taxonomical ones.

[0015] It is therefore the subject of the present invention to present the results of a screening of this microbial diversity and to characterise a microbial genus that would present the characteristics required for a performing cloning host cell: good growth in industrial conditions, transformability, high expression level or well modulated expression level of homologous or heterologous genes or gene sets.

Summary of the invention

[0016] The present invention relates to an isolated *Myrothecium* host cell comprising at least one recombinant DNA construct, or nucleic acid construct, for the modulated expression of homologous genes and/or for the expression of heterologous genes.

[0017] Advantageously, *Myrothecium* host cells were found easy to transform, easy to culture, had a high growth rate coupled to high biomass production and were found to be suitable for growth in fermentors such as for large scale or industrial production of proteins of interest, including but not limited to enzymes such as amylases and xylanases or therapeutic drugs. Applications include but are not limited to protein and/or enzyme production for food and/or therapeutic applications. Another application concerns the use of a genetically engineered (transformed) *Myrothecium* strain as source of biopesticide.

[0018] In an embodiment of the invention, the DNA construct or nucleic acid construct integrates into the host cell chromosome. Alternatively, it may be present on an episome such as a plasmid.

[0019] The DNA construct or nucleic acid construct is built to allow modified expression (such as

overexpression) of a homologous gene and/or may be built to allow expression of a heterologous gene. The protein produced by the transformed *Myrothecium* strain may be a *Myrothecium* protein, a fungal protein as well as a protein normally produced by another organism (i.e. non-fungal). The gene coding for the protein of interest may be engineered and or its codon-use adapted to further increase protein expression or may be engineered to incorporate mutations resulting in altered proteins.

[0020] The DNA construct or nucleic acid construct, if needed, comprises at least one operably linked tool that allows or enhances protein expression, said tool being selected from the group consisting of a promoter, a terminator, a polyadenylation signal, a leader, a secretion signal, a selection marker or reporter gene. Said tool may be of heterologous or homologous origin. It falls within the skills of an artisan to define type, multitude and sense of the genes and/or tools in the expression construct that give rise to optimal protein expression.

[0021] Preferred selection marker genes are the hygromycine B resistance gene, the phleomycin resistance gene, the phosphinothricine resistance gene, the acetamidase gene, a pyrG gene, an argB gene, a niaD gene and a trpC gene.

[0022] A preferred reporter gene is the gene coding for the TAKA-amylase of *Aspergillus oryzae*, optionally associated with flanking regions of the coding region from said TAKA-amylase gene or with flanking regions of the coding region from others genes.

[0023] Preferred promoters are the *Aspergillus oryzae* TAKA-amylase promoter, the *Rhizomucor miehei* aspartic proteinase promoter, the *A. niger* gluocamylase promoter, the *A. niger* neutral α-amylase promoter, the *A. niger* acid stable α-amylase promoter, the *R. miehei* lipase

promoter and the promoters of the glycolytic enzymes genes GPD, PGK and ADH.

[0024] Said promoter may be a regulatable/inducible promoter or a constitutive promoter.

[0025] Suitable Myrothecium sp. include *Myrothecium inundatum*, *Myrothecium prestonii*, *Myrothecium leucotrichum*, *Myrothecium cinctum*, *Myrothecium masonii*, *Myrothecium roridum*, *Myrothecium verrucaria*, *Myrothecium carmichaelii*, *Myrothecium lachastrae*, *Myrothecium atrum*, *Myrothecium atroviride*, *Myrothecium gramineum* (syn. *Xepiculopsis graminea*).

[0026] Especially the *Myrothecium gramineum* strain MUCL39210, the *Myrothecium gramineum* strain CBS449.71, the *Myrothecium gramineum* IMI140595, the *Myrothecium gramineum* IMI290405 and the *Myrothecium verrucaria* strain CBS328.52 were found highly suitable hosts for increased protein production, more in particular large-scale or industrial protein production.

[0027] An embodiment of the invention relates to *Myrothecium* cells transformed to comprise a PCNS43 or a p3SR2 vector.

[0028] The invention further relates to the use of the above-described *Myrothecium* transformants. They are very suitable for the use as cell factory, in particular for the production of proteins and/or enzymes. Possible applications include industrial protein and/or enzyme production, and therapeutical drugs production. Another aspect of the inventions concerns the use of *Myrothecium* transformants as source of biopesticide.

[0029] Another aspect of the present invention concerns methods of transforming *Myrothecium* sp., said methods comprising the steps of growing *Myrothecium* cells or protoplasts; introducing into said cells or protoplasts at least one recombinant DNA construct for the modulated

expression of homologous genes and/or for the expression of heterologous genes into *Myrothecium* cells; and then selecting transformed i.e. genetically modified *Myrothecium* cells. Suitable transformation techniques include PEG-mediated transformation, electroporation, particle bombardment and/or *Agrobacterium*-mediated transformation. These and other suitable transformation techniques are well known in the art. The DNA construct with which to transform the *Myrothecium* host cells may be a plasmid or a vector.

[0030] Another aspect of the invention relates to isolated transformed or genetically modified *Myrothecium* strains obtainable by a method according to claim 18. These transformants or genetically modified strains preferably are characterized by an increased amylase activity, increased xylanase activity, increased growth rate, increased biomass production and/or reduced protease production. Advantageously, the transformants or genetically modified strains will have, after transformation with a suitable DNA construct or nucleic acid construct, an altered metabolic pathway compared to the non-transformed *Myrothecium* strain.

[0031] A further aspect of the present invention concerns a method for producing a protein of interest, said method comprising the steps of culturing transformed or genetically modified *Myrothecium* host cells of the invention under conditions which permit expression of the protein and then recovering the proteins of interest from said *Myrothecium* culture. Said protein preferably is a fungal protein such as a fungal enzyme but may as well be any other protein that can be expressed in *Myrothecium* sp.

[0032] A still further aspect of the present invention concerns a method for producing the biomass of the transformed or genetically modified *Myrothecium* host cells, said method comprising the steps of culturing said

host cells and recovering them with or without the culture medium. Said method thus optionally comprises a purification step to separate *Myrothecium* cells from culture medium or other compounds.

5

Short description of the drawings

[0033] Figure 1 shows a phylogenetic tree constructed with fungal ITS sequences available in the NCBI database or provided in the present invention.

10 **[0034]** Figure 2 shows a partial nucleotide sequence and its deduced protein sequence of the *Myrothecium gramineum* MUCL39210 glyceraldehyde-3-P dehydrogenase gene

[0035] Figure 3 shows a multiple sequence alignment of four partial GPD protein sequence from the strains 15 MUCL39210, MUCL11831, CBS449.71 and IMI290405.

[0036] Figure 4 shows an agarose gel electrophoresis of the DNA fragments amplified by PCR from *Myrothecium* transformants and corresponding to a part of the *Aspergillus oryzae* α-amylase reporter gene (odd lanes from 20 5 to 13) and to a part of the hygromycin selection gene (even lanes from 4 to 12). Lanes 2-3 and 14-15 correspond respectively to negative and positive controls.

[0037] Figures 5A-E show the 28s rDNA sequence of the strains MUCL39210 (A), MUCL11831 (B), CBS449.71 (C), 25 IMI140595 (D) and IMI290405 (E).

[0038] Figures 6A-E show the ITS sequences of the strains MUCL39210 (A), MUCL11831 (B), CBS449.71 (C), IMI140595 (D) and IMI290405 (E).

[0039] Figures 7A-C show the DNA sequences of the 30 gpd gene of the strains MUCL11831 (A), CBS449.71 (B) and IMI290405 (C).

[0040] Figure 8 shows a consensus sequence of the gpd gene of the strain MUCL39210.

[0041] Figure 9 shows the amino acid sequence of the glyceraldehyde 3-P dehydrogenase of *Myrothecium gramineum* (*Xepiculopis graminea*) MUCL39210.

Detailed description of the invention

[0042] A first aspect of the invention is the screening of a collection of fungi. The collection screened in the present invention is the collection from the MUCL ("Mycothèque de l'Université Catholique de Louvain", Place Croix du Sud, 2, B-1348 Louvain-la-Neuve, Belgium). However, it will be obvious for a person skilled in the art that other public or private microorganisms collections can be screened following the same approach to provide the selected genus with the required properties. Examples of public collections where fungal strains are available are ATCC (American Type Culture Collection - P.O. Box 1549, Manassas, VA 20108, USA), CBS (Centraalbureau voor Schimmelcultures - P.O. Box 85167, 3508 AD Utrecht, The Netherlands), DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH - Mascheroder Weg 1 b, D - 38124 Braunschweig, Germany), IMI (International Mycological Institute - Bakeham Lane, Egham, Surrey TW20 9TY, UK),….

[0043] 3.000 different species or strains of fungi were first selected from the MUCL collection based on several criteria such as but not restricted to their non-pathogenicity or the fact that no transformation for this species has been reported in the scientific literature or in a patent. All these strains were grown in liquid culture and on solid medium. Useful liquid media are rich media containing for example glucose, peptone, yeast extract, malt extract, soy meal,…. Useful solid media are for example PDA (Potato Dextrose Agar), MA (Malt Extract Agar). The first selection criteria were based on the growth rate, the biomass production, the protein production, the enzyme

production (xylanase, amylase and proteases) and the morphology of the fungal strains.

[0044] The growth rate can be usefully determined for example by measuring the rate of increase of the growth diameter of a single colony on a solid medium.

The biomass production can be usefully determined by evaluating for example the dry matter of cells collected by filtration or by centrifugation at various time intervals during the culture.

[0045] The protein production can be usefully determined, using methods well known by persons skilled in the art, by measuring the total amount of proteins in a culture supernatant. Examples of such methods are the Lowry (Lowry O.H. et al. 1951. J. Biol. Chem. 193:265) or the Bradford (Bradford M.M. 1976. Anal. Biochem. 72:248) methods.

[0046] The enzyme production can be usefully determined by measuring the amount of enzyme produced in a culture supernatant using methods well known by persons skilled in the art. Examples of methods of xylanase activity determination are the measure of the release of reducing sugar from xylan (Miller G.L. 1959. Anal. Chem. 31:426) or the measure of the release of coloured compounds from modified substrates (for examples AzoWAX or Xylazyme AX from Megazyme). Amylase activity can be determined for example by measuring the rate of release of reducing sugar from starch (Miller, *op.cit.*) or by using specific commercially available kits (amylase kit from Sigma; Amyl kit from Roche Diagnostics,...). Protease activity can be determined for example using commercially available kits (Azocasein from Megazyme,...).

[0047] The morphology of the fungal strains can be advantageously studied by examining portions of the

cultures with a microscope (light microscope, electronic microscope,...).

[0048] From this screening were retained about one tenth of the 3.000 fungal species or strains. Among the selected strains, about 80 strains were chosen based on their good protein production, about 80 based on their good biomass production, about 60 based on their good amylase production, about 60 based on their good xylanase production and about 10 based on their good or bad protease production.

[0049] In one aspect it may be useful to obtain strains that produce very low levels of proteases. This reduces the risk of proteolytic degradation of proteins of interest possibly produced from introduced heterologous genes. Alternatively, good protease producers might be of interest with the purpose of isolating and using for instance a promoter thereof to enhance protein and/or enzyme production in other strains. In one aspect it may be useful to obtain strains that produce very low levels of proteases. This reduces the risk of proteolytic degradation of proteins of interest possibly produced from introduced heterologous genes. Alternatively, good protease producers might be of interest with the purpose of isolating and using for instance a promoter thereof to enhance protein and/or enzyme production in other strains.

[0050] The selected strains were submitted to a second round of screening in liquid cultures using five different culture media, each one having a particular purpose. The first medium has been selected for maximal protein production, the second for maximal biomass production, the third for the amylase induction, the fourth for xylanase induction and the last one for the protease induction.

[0051] Typical media for protein and/or biomass production are rich media composed for example of carbon

and nitrogen sources such as glucose, malt extract, peptone, soy meal,... A typical medium for amylase production contains usually starch or starch derivatives as enzyme inducer. A typical medium for xylanase production usually contains an hemicellulose-rich inducer such as but not restricted to xylan, wheat straw or wheat bran. A typical medium for protease production is usually enriched in various proteins or amino acids sources.

[0052] The screening criteria retained were the same as for the first step: the growth rate, the biomass production, the protein production, the production of amylase, xylanase and/or protease and the morphology.

[0053] A statistical approach was followed to identify the strains that ranked with a high score for one or several criteria in more that one culture medium. About 50 strains were selected out from this second screening.

[0054] In order to eliminate those strains that would produce any antimicrobial compounds, the production of such compounds by the selected strains has been checked. The inhibition of growth of some bacterial species has been tested using the method described by Färber et al (Färber P. and Geisen R. 1994. Antagonistic activity of the food-related filamentous fungus *Penicillium nalgiovense* by the production of penicillin, Appl. Environ. Microbiol. 60:3401-3404). Indicator strains were the following: *Micrococcus flavius* (MUCL collection, determined with API 20E), *Bacillus subtilis* DSM6633, *Bacillus cereus* LMG6923, *Staphylococcus aureus*, *Salmonella* sp. (MUCL collection, determined with API 20E), *Escherichia coli*, *Proteus mirabilis* (MUCL collection, determined with API 20E), *Candida blankii* MUCL29808. These strains have been chosen according to Färber et al (op. cit.), to Aissaoui & al (Aissaoui H., Agut M., Torras M. 1999. Effect of the raw extract of *Arthrinium* strains (Hyphomycetes, Dematiaceae)

on the growth of pathogenic bacteria in poultry feed. Microbios. 100(396):109-115) and to the Oxoid Manual (7th edition 1995). It is also obvious for a person skilled in the art that other bacterial species or strains can equally be used for the evaluation.

[0055] Potential producers of antibacterial compounds have been identified and discarded from the list of selected strains.

[0056] A similar method has been used to control the production of antifungal compounds using *Botrytis cinerea* MUCL30834, *Fusarium moniliforme* MULC14280 and *Alternaria alternata* MUCL16089 (Mangiarotti A., Frate G., Picco AM., Caretta G. 1987. Antagonistic activity *in vitro* of some saprophytic fungi occurring on the phylloplane of rice, wheat and maize. Boletin Micologico. 3:183-189). Again the potential antifungal compounds producers were discarded from the list.

[0057] 30 of the remaining strains (the best ranking in the statistical analysis) were then tested for their ability to incorporate foreign DNA as well as to express a reporter gene. The general method of protoplasts transformation (Punt P.J. and van den Hondel C.A.M.J.J. 1992. Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers, Methods in Enzymology. 216:447-457) has been followed. For each strain several parameters, that a person skilled in the art will recognise as critical, were investigated. Among these parameters were:

- the possibility to use one or several of the common transformation markers (hygromycine B resistance gene, phleomycin resistance gene, phosphinothricine resistance gene, acetamidase gene, pyrG gene, argB gene, niaD gene, trpC...)

- the conditions to obtain viable protoplasts in large amounts (culture medium, culture temperature, length of the culture, type of digesting enzymes, duration of cell walls digestion, type of osmotic stabiliser,…
- the conditions of transformation.
- the conditions of protoplasts regeneration.

[0058] A useful reporter gene is the well-known gene coding for the TAKA-amylase of *Aspergillus oryzae*. The flanking regions of the coding region (essentially the promoter and the terminator sequences) or flanking regions of the coding region from others genes are advantageously associated with the coding region to provide a complete expression cassette. The sequence of the TAKA-amylase gene is publicly available (Tada,S., Iimura,Y., Gomi,K., Takahashi,K., Hara,S. and Yoshizawa,K. 1989. Cloning and nucleotide sequence of the genomic Taka-amylase A gene of *Aspergillus oryzae*. Agric. Biol. Chem. 53:593-599) and the corresponding DNA can be easily cloned using for example PCR techniques.

[0059] It is therefore the object of the present invention to show that the genus *Myrothecium* presents all the characteristics needed for a performing cloning host such as but not limited to good transformability, good expression of homologous and heterologous genes or genes sets, easy metabolic pathway engineering, good performances in fermentation, ….

[0060] In a specific aspect of this embodiment, it is more precisely shown that the species *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) presents all the characteristics needed for a performing cloning host such as but not limited to good transformability, good expression of homologous and heterologous genes or genes sets, easy metabolic pathway engineering, good performances in fermentation, ….

[0061] In a more specific aspect of this embodiment, it is more precisely shown that the strain *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 presents all the characteristics needed for a performing cloning host such as but not limited to good transformability, good expression of homologous and heterologous genes or genes sets, easy metabolic pathway engineering, good performances in fermentation, ….

[0062] The genus *Myrothecium* is defined in the following way:

"Fructification cupulate, sporodochial or synnematous, formed from closely compacted conidiophores arising from a more or less developed stroma and bearing a mass of slimy green to black spores which becomes hard on drying. Fructification surrounded by differentiated marginal hyphae which may be free or laterally compacted into a plectenchymatous wall. Hyaline or darkened setae sometimes present arising from the basal stroma. Coniodiophores hyaline, olivaceous or slightly darkened, macronematous, irregularly and repeatedly branched forming several branches at each node, the ultimate branches bearing the conidiogenous cells (phialides) in whorls. Phialides hyaline or darkened at the apex, occasionally percurrent sometimes with a flared collarette, compacted into a dense parallel layer. Spores unicellular, hyaline or dilute olivaceous, black in mass, slimy" (Tulloch M., 1972, The genus *Myrothecium* Tode ex Fr. Mycological Papers n°130, Commonwealth Mycological Institute, Kew, Surrey, England).

[0063] The genus *Myrothecium* includes *i.a.* the species *Myrothecium inundatum*, *Myrothecium prestonii*, *Myrothecium leucotrichum*, *Myrothecium cinctum*, *Myrothecium masonii*, *Myrothecium roridum*, *Myrothecium verrucaria*, *Myrothecium carmichaelii*, *Myrothecium lachastrae*,

Myrothecium atrum, *Myrothecium atroviride*, *Myrothecium gramineum*,....

[0064] The species *Myrothecium gramineum* or its synonymous species *Xepiculopsis graminea* has the following description:

"Foliicolous. Conidiomata stromatic, scattered to gregarious, seemingly superficial, discoid to cupulate, excipulate with a dense white fringe of long white setae and sterile elements with coiled apices surrounding a central greenish-black agglutinated conidial mass, 220-450 µm diam., 150-190 µm deep (excluding setae); basal stroma moderately developed, composed of a colourless textura angularis in the lower layers gradually merging with textura prismatica above; excipulum well-developed, of compact and colourless textura prismatica to textura porrecta; marginal hyphae with terminal cells 7-16 x 2-3 µm, of two kinds: those on the inside subcylindrical with blunt apices, olivaceous to fuliginous, irregularly tuberculate, and those on the outside with a subcylindrical, olivaceous to fuliginous, irregularly tuberculate basal part, and a slender, coulourless, smooth, sinuate apical process up to 47 µm long and 1 µm wide.

[0065] Conidiomatal setae sparse arising from outer cells of the excipulum, unbranched, straight or slightly incurved, subulate, thick-walled with continuous narrow lumen, smooth-walled, colourless, up to 450 µm long, 15-20 µm wide at the broadest point, up to 1.5 µm wide at the acute apex. Conidiophores arising from the uppermost cells of the basal stroma and inner layer of cells of the excipulum in the concavity of the conidiomata, unbranched or branched, septate, colourless to almost colourless, smooth. Conidiogenous cells discrete, occasionally integrated, subcylindrical to clavate with flared collarettes and moderate to marked apical periclinal

thickenings, more or less pale olivaceous in the upper part with a deeper pigmentation near the collarettes, smooth, (7-)12-16 x 2-3 [mean = 14 x 2.5] µm. Conidia fusiform to ellipsoid with an acute apex and a narrow truncate base, unicellular, pale olivaceous, smooth, 6.5-12.5 x 2-3(-3.5) [mean = 9 x 2.5] µm, with an apical mucoid appendage; mean conidium length/width ratio = 3.6:1. (NAG RAJ T.R. 1993. Coelomycetous anamorphs with appendage-bearing conidia, published by Mycologue Publication)

[0066] It should be understood that in the present invention the terms *Myrothecium sp.*, *Myrothecium gramineum* or *Xepiculopsis graminea* refer not only to those organisms present currently under these names in the various public or private microorganisms collections around the world but also to the species which have previously been or currently are assigned to other species but that possess the same morphological and cultural characteristics as defined above.

[0067] The scope of the present invention also includes mutant strains of *Myrothecium sp.*, *Myrothecium gramineum* or *Xepiculopsis graminea*. Types of mutants include but are not restricted to auxotrophic mutants, protease(s) deficient mutants, secretion mutants, constitutive mutants, deregulated mutants. Mutants can be obtained using techniques well known by persons skilled in the art.

[0068] In another embodiment of this invention, it will be referred to rDNA sequences or ITS sequences to assign a particular strain to a species or a genus.

[0069] The internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) has been widely used to reconstruct microbial phylogeny. The ITS region separating 18s and 26s nrDNA and the 5.8s coding region sequence has been widely used to characterise interspecific and

intergenic levels divergences. The ITS region is therefore useful to gain insight into DNA sequence evolution. This is due to the fact that, as a transcribed, but untranslated sequence, it is free to vary.

[0070] Using a combination of the morphological taxonomical methods and the ITS sequences determination and comparison, the inventors have shown that some of the strains deposited as *Myrothecium gramineum* (or as its synonymous *Xepiculopsis graminea*) in public culture collections have been erroneously identified as *Myrothecium gramineum* or *Xepiculopsis graminea*. They have also shown that representative members of other species of the genus *Myrothecium* also present the characteristics needed for a performing cloning host such as but not limited to good transformability, good expression of homologous and heterologous genes or genes sets, easy metabolic pathway engineering, good performances in fermentation, ….

[0071] Other species of the genus *Myrothecium* are therefore considered as integral part of the present invention.

[0072] *Aspergillus* species and in particular *Aspergillus niger* have been used since years as cell factories to produce homologous and heterologous proteins and can therefore be considered as the models for gene expression in filamentous fungi (see for example the review van Gorcom R.F.M., Punt P.J., van den Hondel C.A.M.J.J. 1994. Heterologous gene expression in *Aspergillus* in The genus Aspergillus: from taxonomy and genetics to industrial applications Powell K.A., Renwick, A., Peberdy J.F. eds. Plenum Press, New York, pp 241-250).

[0073] It is therefore one of the objects of the present invention to show that strains from the selected filamentous fungi genus produce, when transformed with

homologous or heterologous DNA more protein and/or enzyme than *Aspergillus niger* in the same conditions.

[0074] It is a further object of the present invention to provide nucleic acid or DNA constructs (recombinant DNA constructs) that, when used to transform *Myrothecium sp.*, *Myrothecium gramineum* or *Xepiculopsis graminea*, allow the expression of the homologous or the heterologous gene present on this nucleic acid or DNA construct. The foreign (introduced) DNA may integrate into the host genome or may be present on an episome. A particular result of the present invention is that the DNA when introduced in the host cell, integrates in the host cell chromosome. This phenomenon is similar to what has already been described for many other species of fungi. Therefore, it is obvious for a person skilled in the art that the same type of constructs as the one used for other species of filamentous fungi can be used for *Myrothecium sp.*, *Myrothecium gramineum* or *Xepiculopsis graminea*. For example, the DNA constructs that are useful in the transformation of *Aspergillus* species such as *Aspergillus niger*, *Aspergillus nidulans* or *Aspergillus oryzae* are also useful for the purpose of the present invention. The promoter may be any DNA sequence that induces a transcriptional activity in the host and may be derived from any homologous or heterologous gene. Examples of promoters are the *Aspergillus oryzae* TAKA-amylase promoter, the *Rhizomucor miehei* aspartic proteinase promoter, the *A. niger* gluocamylase promoter, the *A. niger* neutral α-amylase promoter, the *A. niger* acid stable α-amylase promoter, the *R. miehei* lipase promoter and the promoters of glycolytic enzymes genes such as GPD, PGK, ADH. These promoters may be regulatable/inducible or not. Transcription terminator and polyadenylation sequences may also be added to the nucleic acid or DNA constructs.

[0075] In a particular aspect of this embodiment, it is also provided additional tools to enhance the gene expression in the selected host strains, namely *Myrothecium sp.*, *Myrothecium gramineum*, or *Xepiculopsis graminea*. Using methods well known by persons skilled in the art, the gene coding for the glyceraldehyde 3-phosphate dehydrogenase (GPD gene) has been partially cloned. Flanking regions (promoter and terminator) of these sequences highly suited to make nucleic acid or DNA constructs that give a better expression in the selected host strains.

[0076] In a further aspect of this embodiment, the nucleic acid or DNA constructs may comprise particular sequences, such as sequences coding for a leader or a signal peptide, that allows the protein of interest to be secreted in the extracellular medium. This preregion may be derived from any secreted protein, or may be synthesised using consensus sequences, provided that this preregion allows the secretion of the desired protein.

[0077] In yet another aspect of this embodiment, the nucleic acid or DNA constructs may comprise genes to allow the selection of the transformants of *Myrothecium sp.*, *Myrothecium gramineum* or *Xepiculopsis graminea*. Those selection markers are for example but are not restricted to the acetamidase gene (amdS) of *Aspergillus* sp. or the hygromycine resistance gene (hygB) of *Escherichia coli*. Other markers include the argB, niaD, trpC or pyrG genes of *Aspergillus sp.*

[0078] In yet another aspect of this embodiment, the various elements of the DNA constructs may be either incorporated in the same vector or plasmid or DNA fragment or may be included in separate vectors, plasmids or DNA fragments provided that the DNA sequences required for gene expression are associated with their respective coding sequence.

[0079] An example of suitable selection vector is the plasmid pCSN43 that contains the hph gene of *Escherichia coli* under the control of *Aspergillus* transcriptional signals (Staben C. et al. 1989. Use of a bacterial hygromycine B resistance gene as a dominant selectable marker in *Neurospora crassa* transformation. Fungal Genetics Newsl. 36:79). It confers to transformed or genetically modified cells resistance to hygromycine B.

[0080] Another example of suitable selection vector is the plasmid p3SR2 that contains the acetamidase gene from *Aspergillus nidulans* (Hynes et al. 1983. Isolation of genomic clones containing the amdS gene of *Aspergillus nidulans* and their use in the analysis of structural and regulatory mutations. Mol. Cell. Biol. 3:1430). It confers to transformed or genetically modified cells the ability to grow on a minimal medium containing acetamide as sole nitrogen source.

[0081] In another embodiment of the present invention, a method is provided to transform *Myrothecium sp.*, *Myrothecium gramineum* or *Xepiculopsis graminea*. The preferred method of transformation is the standard protoplast transformation. In this method, the DNA constructs are incubated in particular conditions with protoplasts of the host strain. The transformants are first selected on the basis of the selection marker used (for example amdS or hygB) and in a second step cotransformants that have integrated the desired gene are screened.

It is one of the objects of the present invention to show that, when transformed or genetically modified with two separate DNA constructs (containing for example a selection marker and a reporter gene), most of the transformants of *Myrothecium*, *Myrothecium gramineum* or *Xepiculopsis graminea* have integrated simultaneously both DNA constructs.

[0082] Other methods of transformation can usefully replace the protoplasts transformation and are well known by persons skilled in the art. Examples of alternative methods are the electroporation, the transformation by particle bombardment or the transformation by *Agrobacterium* sp.-mediated recombination.

[0083] In another embodiment of the present invention, it is provided *Myrothecium sp.*, *Myrothecium gramineum* or *Xepiculopsis graminea* transformed or genetically modified strains that express or overexpress a particular homologous or heterologous gene or a particular set of genes.

[0084] In another embodiment of the present invention, it is provided *Myrothecium* sp. *Myrothecium gramineum* or *Xepiculopsis graminea* genetically engineered strains with one or several altered metabolic pathways.

[0085] In another embodiment of the present invention it is provided culture media to cultivate the *Myrothecium sp.*, *Myrothecium gramineum* or *Xepiculopsis graminea* transformed or genetically modified strains. A typical culture media contain carbon (C) sources and/or nitrogen (N) sources and/or salts and/or secondary metabolites and/or miscellaneous compounds. C sources could be glucose, saccharose, … as well as complex substrates like arabinoxylan, pectin, … as well as lipids and organic acids. N sources could be amino acids, nitrate, ammonia, … as well as complex substrates like yeast extract, potato extract, soy extract, ….

[0086] Salts could be chosen among phosphate salts, Magnesium salts, potassium salts, …. Secondary metabolites could be antibiotics, mycotoxins, hormones, vitamins,…. Miscellaneous compound could be defoamers, antifoamers, emulsifiers, ….

[0087] In another embodiment of the present invention it is provided culture techniques to cultivate the *Myrothecium sp.*, *Myrothecium gramineum* or *Xepiculopis graminea* or genetically modified strains. Examples of such culture techniques are liquid cultures in erlenmeyer flasks or fermentor, solid state fermentation, airlift fermentation,...

[0088] This invention will be described in further details in the following examples by reference to the enclosed drawings, which are not in any way intended to limit the scope of the invention as claimed.

EXAMPLE 1

Characterisation of the *Myrothecium* strains

1.1. Molecular characterisation of the Myrothecium strains

[0089] The genomic DNA of the strains currently assigned as *Myrothecium gramineum* (or of the synonymous strain *Xepiculopsis graminea*) MUCL39210, MUCL11831, CBS449.71, IMI140595 and IMI290405 has been prepared using the Dneasy Plant Mini Kit (Qiagen).

[0090] The DNA coding for RNA ITS (Internal Transcribed Spacer) and 28S regions were analysed by PCR. The oligonucleotides used for the 28S region were: LR0R (5'-ACCCGCTGAACTTAAGC-3', SEQ ID No 1), LR6 (5'-CGCCAGTTCTGCTTACC-3', SEQ ID No 2), LR3R (5'-GTCTTGAAACACGGACC-3', SEQ ID No 3) and LR3 (5'-CCGTGTTTCAAGACGGG-3', SEQ ID No 4). The oligonucleotides used for the ITS region were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3', SEQ ID No 5) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3', SEQ ID No 6).

[0091] PCR reactions were performed in 0.25 ml tubes, using a MJ Research PTC-200 Peltier Thermal Cycler. PCR reactions (50 µl) contained approximately 20 ng of

fungal genomic DNA (in 10 µl), 10 pM ITS1 and ITS4 primer pairs (1 ml), 100 mM dNTPs (1 µl), 1X reaction buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl.sub.2, 50 mM KCl, 0.1 ng/ml BSA) (5 µl), and 2.5 U Taq polymerase (Invitrogen) (0.5 µl). Thermal cycling conditions were as follows: for 28S: initial denaturation (94°C, 5 min), followed by 35 cycles of denaturation (94°C, 1 min), annealing (50°C, 1 min 30 s), primer extension (72°C, 4 min), and one final cycle of primer extension (72°C, 10 min); for ITS: initial denaturation (94°C, 3 min), followed by 30 cycles of denaturation (94°C, 1 min 30 s), annealing (55°C, 1 min 30 s), primer extension (72°C, 2 min), and one final cycle of primer extension (72°C, 10 min).

[0092] The reaction products (10 µl) were analysed by electrophoresis on a 0.8 % agarose gel in Tris-acetate EDTA buffer (TAE buffer) to check the presence of a unique amplified DNA fragment. The PCR product was then purified using PCR purification kit (Qiagen) and quantified with a Biophotometer (Eppendorf).

[0093] DNA sequencing reactions were performed in 0.25 ml tubes, using a MJ Research PTC-200 Peltier Thermal Cycler. PCR reactions (20 µl) contained 25 to 100 fmol of purified PCR product (0.5 to 10 µl), 1.6 µM of -47 sequencing primer (2 µl), 8 µl of DTCS Quick Start Master Mix (100 µl of dNTPs, 200 µ l of ddATP, 200 µl of ddGTP, 200 µl of ddCTP, 200 µl of ddUTP, 200 µl of 10X reaction buffer and 100 µl Taq polymerase). Thermal cycling conditions were as follows: 30 cycles of denaturation (96°C, 20 sec), annealing (50°C, 20 sec), primer extension (60°C, 4 min), followed by holding at 4°C. The reaction is stopped by adding 4 µl of Stop Solution (1.5M NaOAc + 50 mM EDTA prepared fresh daily by mixing equal volumes of 3M NaOAc and 100mM EDTA) and 1 µl of 20 mg/ml glycogen. The sequencing reactions were transferred to a 96 wells plate.

60 µl of 95% cold ethanol from -20°C freezer were added, the plate was sealed with adhesive foil, mixed thoroughly, incubated at -20°C during 10 minutes, centrifuged at 14.000 rpm at 4°C during 30 minutes and the supernatant was carefully removed. The pellets were then rinsed two times with 200 µl of 70% cold ethanol. Finally, the samples in the plate were dried under vacuum until no traces of ethanol remained (5 to 15 minutes). The samples were resuspended in 40 µl of Sample Loading Solution, left at room temperature for ten minutes then mixed thoroughly and overlaid with one drop of light mineral oil (Sigma Cat# M 5904).

[0094] The sequencing was done in a capillary electrophoresis sequencer "CEQ™ 2000 XL DNA Analysis System" (Beckman Coulter). The sequences generated were rectified and aligned with the software Sequencher™ 4.1. The sequence of 28s rDNA of the strain MUCL39210 is shown in SEQ ID No 7.

[0095] The sequence of 28s rDNA of the strain MUCL11831 is shown in SEQ ID No 8.

[0096] The sequence of 28s rDNA of the strain CBS449.71 is shown in SEQ ID No 9.

[0097] The sequence of 28s rDNA of the strain IMI140595 is shown in SEQ ID No 10.

[0098] The sequence of 28s rDNA of the strain IMI290405 is shown in SEQ ID No 11.

[0099] The ITS sequence of the strain MUCL39210 is shown in SEQ ID No 12.

[0100] The ITS sequence of the strain MUCL11831 is shown in SEQ ID No 13.

[0101] The ITS sequence of the strain CBS449.71 is shown in SEQ ID No 14.

[0102] The ITS sequence of the strain IMI140595 is shown in SEQ ID No 15.

[0103] The ITS sequence of the strain IMI290405 is shown in SEQ ID No 16.

[0104] These sequences were compared and compared with other closely related sequences of the NCBI gene bank (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences were first aligned using the software ClustalX 1.5b and then a bootstrap tree was constructed using the software PAUP 4.0b10.

[0105] A phylogenetic tree with relevant data is presented on figure 1. It is concluded from this tree that some of the investigated *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) strains have been erroneously assigned to a particular species. However, all the strains studied, with the exception of MUCL11831 belong to the *Myrothecium* genus.

1.2. Morphological identification of the Myrothecium strains.

[0106] A taxonomical reidentification of the MUCL39210 strain has confirmed that it presents all the morphological characteristics of the species *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) (see the description in the detailed description of the invention).

[0107] A similar examination of the strain MUCL11831 confirmed that this strain does not belong to the *Myrothecium* genus.

EXAMPLE 2

Transformation of *Myrothecium*

2.1. Choice of selectable markers

[0108] *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 was incubated on PDA (Potato Dextrose Agar), AMMA (Aspergillus Minimal Medium Agar) or MA2 (Malt

Extract Agar) plates with increasing concentration of hygromycin B, phleomycin or phosphinothricine. The threshold antibiotic concentration where no growth occurred was 500 µg/ml for hygromycin B, >100 µg/ml for phleomycin and > 400µg/ml for phosphinothricin.

[0109] *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 was incubated on minimal medium plates containing acetamide as sole nitrogen source. The strain did not grow on this medium.

[0110] Similar results were obtained with the strains of *Myrothecicum gramineum* CBS449.71, IMI140595, IMI290405 and *Myrothecium verrucaria* CBS328.52

2.2. Vectors

2.2.a. *Selectable vector*

[0111] The vector chosen to allow the selection of the transformants by their resistance to an antibiotic is the plasmid pCSN43 that contains the hph gene of *Escherichia coli* under the control of *Aspergillus* transcriptional signals (Staben C. et al. 1989. Use of a bacterial hygromycine B resistance gene as a dominant selectable marker in *Neurospora crassa* transformation. Fungal Genetics Newsl. 36:79).

[0112] The vector chosen to allow the selection of the transformed or genetically modified cells by their ability to grow on a minimal medium containing acetamide as sole nitrogen source is the plasmid p3SR2 (Hynes *et al.* 1983. Isolation of genomic clones containing the amdS gene of *Aspergillus nidulans* and their use in the analysis of structural and regulatory mutations. Mol. Cell. Biol., 3:1430)

2.2.b. *Expression vectors*

2.2.b.1. p2G-S

[0113] A DNA fragment covering the coding region as well as its terminator region was amplified by a PCR reaction. The synthetic oligonucleotide AMY1 (5'-GGAATTCCACAGAAGGCATTTATG-3', SEQ ID No 17) was chosen to contain the ATG codon of the first amylase methionine. The synthetic oligonucleotide AMY2 (5'-GCTCTAGAGCAACCACCAGGTCA-3', SEQ ID No 18) corresponds to the sequence located about 400 bp downstream of the amylase terminal codon.

[0114] The two oligonucleotides have sequences recognised respectively by the restriction enzymes *Eco*RI and *Xba*I. Both primers (40 pmoles) were used for a PCR reaction with the gDNA from *Aspergillus oryzae* MUCL14492. The PCR reaction contained 2.5 U *Pfu* DNA polymerase (Stratagene), 20 mM Tris-HCl pH 8.0, 10 mM KCl, 2 mM $MgCl_2$, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 1 µg BSA into a final volume of 100 µl. After denaturation of the DNA during 4 min at 94°C, the reaction was performed with 20 cycles of 30 s at 94°C, 30 s at 55°C and 60 s at 72°C followed by an elongation step of 7 min at 72°C. The amplified DNA fragments were purified in a final volume of 50 µl with the QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol. The purified fragment was treated with the *Eco*RI and *Xba*I restriction enzymes. The reaction was performed with 5 U Xba I and 5 U EcoR I (Pharmacia), 1 x One-Phor-All buffer PLUS in 60 µl at 37°C overnight. The restricted fragments were purified with the QIAquick gel extraction kit (Qiagen) after electrophoresis on an agarose gel and eluted into 30 µl water.

[0115] The PCR fragment was inserted between the *Eco*R I and *Xba* I sites of the pBluescript II SK(-) vector (Stratagene). The vector was prepared as follows:. 0.5 µg

pBluescript II SK was treated by 5 U *Eco*R I and 5 U *Xba* I restriction enzymes (Pharmacia) in a 20 µl final volume, 2 x One-Phor-All buffer PLUS at 37°C overnight. After electrophoresis in an agarose gel, it was purified with QIAquick gel extraction kit (Qiagen) and eluted in 30 µl water. 2 µl of the PCR fragment was ligated to this vector (1 µl) in the presence of 1 mM ATP, 1U T4 DNA ligase (Pharmacia) and 1 x One-Phor-All buffer PLUS in a 10 µl final volume at 16°C overnight. The ligation product (1µl) was electroporated into competent DH10b cells (BRL-Gibco) after dialysis against water. The new plasmid was termed pAMY3.

[0116] The promoter of the glyceraldehyde-3-P dehydrogenase gene from *Aspergillus nidulans* was inserted in front of the amylase gene. This promoter allows a rather strong constitutive transcription of genes located downstream of it (Punt, P.J., Dingemanse, M.A., Kuyvenhoven, A., Soede, R.D.M., Pouwels, P.H. and van den Hondel; C.A.M.J.J., 1990. Functional elements in the promoter region of the *Aspergillus nidulans gpd*A gene coding for the glyceraldehyde-3-P dehydrogenase. Gene. 93: 101-109 ; Punt, P.J., Zegers, N.D., Busscher, M., Pouwels, P.H. and van den Hondel; C.A.M.J.J., 1991. Intracellular and extracellular production of proteins in *Aspergillus* under the control of expression signals of the highly expressed *Aspergillus nidulans gpd*A gene. J. Biotechnol. 17: 19-34). To insert the amylase gene downstream of the GPD promoter, the plasmid pFGPDGLAT2 was digested with the restriction enzyme *Nco*I . After purification with the QIAquick gel extraction kit (Qiagen), the plasmid was blunted with T4 DNA polymerase and dNTP, purified, and restricted with *Xba*I restriction enzyme (5U) in the presence of 1 x One-Phor-All buffer PLUS in a 20 µl final volume 37°C overnight). The same protocol was applied on

pAMY3 except that the *Nco*I restriction enzyme was replaced by *Eco*RI. The fragments of interest were isolated after agarose gel electrophoresis, purified with QIAquick gel extraction kit (Qiagen) and collected separately in 30 µl water. The purified promoter and vector DNA fragment (1 µl) was ligated with the amylase gene and its terminator from pAMY3 (1 µl) in the presence of 1 mM ATP, 1U T4 DNA ligase (Pharmacia) and 1 x One-Phor-All buffer PLUS in a 10 µl final volume at 16°C overnight. The ligation product (1µl) was electroporated into competent DH10b cells (BRL-Gibco) after dialysis against water. The new plasmid was named pGPD-AMY.

[0117] The GPD promoter, the amylase gene and its terminator haves been isolated from pGPD-AMY and inserted again into a new pGPD-AMY in order to have two copies of the functional gene on one plasmid. The first step was to digest pGPD-AMY with 1U *Hin*dIII restriction enzyme and 1 x One-Phor-All buffer PLUS in a 40 µl final volume at 37°C overnight. After purification with QIAquick gel extraction kit (Qiagen), the linear DNA fragment was blunted in the presence of T4 DNA polymerase and dNTP. The plasmid was again purified with QIAquick gel extraction kit (Qiagen) and treated with *Not*I restriction enzyme and 1 x One-Phor-All buffer PLUS in a 40 µl final volume at 37°C overnight. The fragment containing the functional amylase gene was finally isolated on agarose gel after electrophoresis. The pGPD-AMY plasmid (1µg) was digested by *Xba*I (1U) and 1 x One-Phor-All buffer PLUS in a 40 µl final volume at 37°C overnight. After purification with QIAquick gel extraction kit (Qiagen), the linear DNA fragment was blunted in the presence of T4 DNA polymerase and dNTP. The plasmid was again purified with QIAquick gel extraction kit (Qiagen) and digested with the *Not*I restriction enzyme and 1 x One-Phor-All buffer PLUS in a 40 µl final volume at 37°C

overnight. The vector with the DNA fragment containing a functional amylase gene was isolated after agarose gel electrophoresis. Both fragments were ligated together in the presence of 1 mM ATP, 1U T4 DNA ligase (Pharmacia) and 1 x One-Phor-All buffer PLUS in a 10 µl final volume at 16°C overnight. The ligation product (1µl) was electroporated into competent DH10b cells (BRL-Gibco) after dialysis against water. The new plasmid was termed p2GPD-AMY.

[0118] Finally a selection marker was inserted in p2GPD-AMY. The acetamidase gene will allow the growth of fungi on a minimal medium with acetamide as unique nitrogen source. The functional acetamidase gene from *A. nidulans* (AmdS) has been isolated from 1 µg of p3SR2 plasmid digested with the restriction enzymes*Sal*I (1U) and *Eco*RI (1U) and 2 x One-Phor-All buffer PLUS in a 40 µl final volume at 37°C overnight. This DNA fragment was inserted between the *Sal*I and *Eco*RI restriction sites of pBluescript II SK. This new plasmid as well as p2GPD-AMYwere digested with *Sal*I (or Xba I for p2GPD-AMY), blunted by treatment with the T4 DNA polymerase and digested with *Not*I. The functional AmdS gene DNA fragment and the openp2GPD-AMY plasmid were isolated from agarose after gel electrophoresis. Both fragments were then ligated together in the presence of 1 mM ATP, 1U T4 DNA ligase (Pharmacia) and 1 x One-Phor-All buffer PLUS in a 10 µl final volume at 16°C overnight. The ligation product (1µl) was electroporated into competent DH10b cells (BRL-Gibco) after dialysis against water. The new plasmid was termed p2G-S.

2.2.b.2. Cloning of the GPD promoter

Design of the oligonucleotides

[0119] Based on published sequences of fungal glyceraldehyde-3-P dehydrogenases (gpd), a set a four oligonucleotides has been designed in order to amplify a portion of the gpd gene of *Myrothecium*.

These oligonucleotides have the following sequences:

Gpd1 : 5'-GGNATCAAYGGITTCGG-3', SEQ ID No 19

Gpd2 : 5'-GTGSWGSWGGGGATGATGTT-3', SEQ ID NO 20

Gpd3 : 5'-GGTCGTATCGTNTTYCGIAAYGC-3', SEQ ID No 21

Gpd4 : 5'-GGAGCCAGGCAGTTGGTIGTRCA-3', SEQ ID No 22

Where N stands for A, T, C or G; Y stands for C or T; I stands for dInosine; S stands for G or C; W stands for A or T; R stands for A or G.

Preparation of the genomic DNA

[0120] The genomic DNA of the following strains has been prepared using the Dneasy Plant Mini Kit (Qiagen): MUCL39210, MUCL11831, CBS449.71, IMI140595 and IMI290405.

PCR

[0121] PCR reactions have been performed in 0.25 ml tubes with a MJ research PTC-200 Peltier Thermal Cycler. The reaction mix (25 μl) contained about 20 ng genomic DNA, 10 pM oligonucleotides, dNTP 200 μM, 1 x buffer (Tris-HCl 10 mM, pH 8.3, $MgCl_2$ 1.5 mM, KCl 50 mM, BSA 0.1 ng/ml) and 1 unit of Taq polymerase (Invitrogen). Amplification conditions were the following: initial denaturation (94°C, 4 min); 25 cycles of denaturation (94°C, 30 sec)-hybridisation (52°C, 30 sec)-elongation (72°C, 1 min); final elongation (72°C, 7 min).

[0122] Cloning of the gpd gene of the strains MUCL11831, CBS449.71 and IMI290405.

[0123] DNA fragments amplified using the protocol described above (with the oligonucleotides gpd1 and gpd2 as amplification primers) have been separated by agarose gel electrophoresis and purified with the aid of a QIAquick Gel Extraction Kit (Qiagen). A second round of PCR has been performed on these purified fragments and the resulting DNA fragments have been directly ligated into the plasmid commercially available pT-Adv (Advantage PCR cloning Kit - Clontech) using the recommendations of the manufacturer. Ligations products have been used to transform competent cells of *Escherichia coli* DH5α (Invitrogen). Positive clones have been identified by plasmid isolation and analysis by restriction enzyme digestion and agarose gel electrophoresis.

[0124] Sequencing of the gpd genes of the strains MUCL11831, CBS449.71 and IMI290405.

[0125] The insert of the plasmid described above has been sequenced using usual procedures.

[0126] The sequence of the gpd gene of the strain MUCL11831 is shown in SEQ ID No 23.

[0127] The sequence of the gpd gene of the strain CBS449.71 is shown in SEQ ID No 24.

[0128] The sequence of the gpd gene of the strain IMI290405 is shown in SEQ ID No 25.

Cloning of the gpd gene of MUCL39210

[0129] The same procedure as for the cloning of the gpd genes of the strains MUCL11831, CBS449.71 and IMI290405, except that the first PCR was performed using the oligonucleotides gpd2 and gpd3 and that the second PCR was performed with the oligonucleotides gpd3 and gpd4.

Sequencing of the gpd gene of the strain MUCL39210.

[0130] The inserts of 8 different inserts of the plasmids obtained above have been sequenced using usual procedure.

[0131] A consensus sequence of the gpd gene of the strain MUCL39210 is shown in SEQ ID No 26.

Results

[0132] The MUCL39210 GPD partial sequence shown in SEQ ID NO14 was translated into a putative protein sequence as presented in Figure 2. The GPD partial sequence comprises a large putative intron. The intron sequence does not show homology to any protein from protein databases when translated from the six possible DNA frames. The putative protein sequence (SEQ ID No 27) shows the highest similarity with the GPD protein sequence of *Neurospora crassa* using the BLASTP 2.2.4. program against the non-redundant GenBank database as of 20th Augustus 2002. Between the various *Myrothecium* GPD protein sequences, the identity is 88% as shown in figure 3.

2.3. Transformation

2.3.a. *Transformation of Myrothecium*

[0133] The strains *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210, *Myrothecium gramineum* CBS449.71, IMI140595, IMI290405, and *Myrothecium verrucaria* CBS328.52 were transformed by generating protoplasts according to the protocol described by Punt et al. (Punt *et al.* 1992. Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. Meth. Enzymol. 216:447).

[0134] The strains were grown in 350 ml Rich Liquid medium (malt extract 2%, peptone 1% and glucose 3%) at 25°C

for a period between 16 and 36 hours depending on the strain. The culture was filtered through a Miracloth filter to collect the mycelium. The mycelium was washed with the OSM solution ($CaCl_2$ 0.27 M, NaCl 0.6 M) and then incubated with 20 ml OSM solution/g mycelium supplemented with 12 mg/ml β-D-glucanase (Interspex), 7.5 mg/ml driselase (Interspex) and 81.4 U/ml yeast lytic enzyme (ICN). The incubation is done at 25°C with slow agitation (80 rpm).

[0135] The apparition of the protoplasts is followed visually under the microscope and varies from 90 to 180 min, depending on the strain. Once the protoplasts were formed, the suspension was putted on ice. The protoplasts were separated from intact mycelium by filtration through a sterile Miracloth filter and diluted with 1 volume STC1700 solution (sorbitol 1.2 M, Tris-HCl pH 7.5 10 mM, $CaCl_2$ 50 mM, NaCl 35 mM) or GTC (glucose 1 M, $CaCl_2$ 50 mM, Tris 10 mM pH8). The protoplasts were then collected by centrifugation at 2000 rpm, 10 min, 4°C and washed twice with STC1700 or GTC solution. They were finally resuspended in 100 µl of STC1700 or GTC (10^8 protoplasts/ml).

[0136] To obtain transformants resistant to hygromycine, c.a. 2 µg pCSN43 plasmid DNA and c.a. 8 µg p2G-S plasmid DNA were added to the protoplasts suspension. To obtain transformants able to grow on acetamide as sole nitrogen source 3 µg p3SR2 plasmid DNA and 9 µg p2G-S plasmid DNA were added to the protoplasts suspension.

[0137] After 30 min at 20°C, 250, 250 and 850 µl PEG solution (PEG 4000 60%, Tris-HCl 10 mM pH 7.5 and $CaCl_2$ 50 mM) were added successively and the suspension was further incubated for 20 min at 20°C. PEG treated protoplasts suspension was diluted by the addition of 50 ml STC1700 or GTC and centrifuged 5 min at 4°C, 2000 rpm. The protoplasts

were then resuspended in 200 µl STC 1700 or GTC and plated onto selective medium.

[0138] For hygromycin selection Malt Extract Agar Medium or Potato Dextrose Agar osmotically stabilised with 1.2 M sorbitol or 1 M glucose and supplemented with 500 µg/ml hygromycin was used. In some cases, protoplasts are plated onto the same medium without hygromycin. After 2 hours incubation, hygromycin (500 µg/l) overlays (8 ml molten agar) were dispensed over the protoplasts.

[0139] For acetamidase selection the AMM medium (Pontecorvo, G., Roper, J.A., Hemmons, L.M., Mc Donald, K.D. and Bufton, A.W.J., 1953. Adv. Genet. 5: 142-152.) supplemented with sorbitol 1.2M and acetamide as sole nitrogen source was used.

[0140] A series of transformants has been obtained for each strain and for each transformation trial. These transformants have been replicated on the same selection medium without sorbitol and kept for further analysis.

2.3.b. *Control transformation.*

[0141] The same protocol as described in 2.3.a. has been used to transform the MUCL28817 strain of *Aspergillus niger*. A number of transformants have been selected and kept for further analysis.

EXAMPLE 3.

Analysis of the transformants

3.1. Amplification of integrated DNA

3.1.a. *Preparation of genomic DNA from fungal strains*

[0142] The genomic DNA of the transformed or genetically modified fungal strains as well as the control strains has been prepared using the Dneasy Plant Mini Kit (Qiagen)

3.1.b. *PCR reactions*

[0143] According to the published sequence of the *E. coli* hygromycin resistance gene (hph gene), the oligonucleotides hphpCSN431 (5'-ATGCCTGAACTCACCGCGACG-3', SEQ ID No 28) and hphpCSN432 (5'-CTATTCCTTTGCCCTCGG-3', SEQ ID No 29) were designed to amplify a region of 1020 bp comprising the hph gene.

[0144] To detect the presence of p2G-S into the *Myrothecium* genome without amplification of the endogenous amylase gene or the GPD promoter, the GPD2 (5'-TCTGGCATGCGGAGAG-3', SEQ ID No 30) and AMY5 (5'-CGATGATGCCCTGCCA-3', SEQ ID No 31) oligonucleotides were designed to allow the amplification of a 1134 bp length fragment covering a part of the *A. niger* GPD promoter fused to a part of the sequence of the TAKA amylase gene.

[0145] PCR reactions have been performed in 0.25 ml tubes with a MJ research PTC-200 Peltier Thermal Cycler. The reaction mix (25 µl) contained about 20 ng genomic DNA, 10 pM oligonucleotides, dNTP 200 µM, 1 x buffer (Tris-HCl 10 mM, pH 8.3, $MgCl_2$ 1.5 mM, KCl 50 mM, BSA 0.1 ng/ml) and 1 unit of Taq polymerase (Invitrogen). Amplification conditions were the following: initial denaturation (94°C, 4 min); 25 cycles of denaturation (94°C, 30 sec)-hybridisation (55°C, 30 sec)-elongation (72°C, 1 min); final elongation (72°C, 7 min).

3.1.c. *Results*

[0146] The results of the analysis of 5 different transformants of *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 selected for the hygromycin resistance and amylase production are shown on figure 4. The agarose gel electrophoresis of the PCR products shows that all the five transformants studied have integrated both the selection marker gene (hph gene - c.a. 1000 bp DNA fragment

- lanes 4, 6, 8, 10 & 12) and the reporter gene (α-amylase gene of *Aspergillus oryzae* - c.a. 1100 bp DNA fragment - lanes 5, 7, 9, 11 & 13). Negative controls are presented in the lanes 2 & 3 (respectively products of hygromycine and α-amylase genes PCR amplification). Lanes 14 and 15 are positive controls (PCR reactions performed respectively on the pCSN43 and p2G-S plasmids).

3.2. Evaluation of recombinant protein production

3.2.a. *Culture of the transformants*

[0147] Twelve transformants of *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 obtained by transformation with the plasmids pCSN43 and p2G-S as well as 49 transformants of *Aspergillus niger* MUCL28817 have been cultivated in AMM medium with 3% sucrose as carbon source in shaking incubators (140 rpm) at 25°C. The culture volume was 20 ml for a total erlenmeyer volume of 100 ml. After 4 days, the supernatants of the cultures were collected by centrifugation.

3.2.b. *α-amylase activity determination*

[0148] The α-amylase activity was determined by a modification of the method included in the Sigma Amylase kit. The substrate was diluted in a 100 mM NaH_2PO_4 buffer pH 6.0. One amylase unit was defined as the amount of enzyme that gives an absorbance of 1 OD unit at 405 nm after 15 min reaction at 37°c and when 120 µl of the reaction mix are red in a 96 holes microplate with the model 550 microplate reader (Bio-Rad).

3.2.c. *Biomass determination (Dry matter determination)*

[0149] The amount of biomass in a culture was determined by filtering an aliquot of the culture through a

Whatman n°540 filter and putting the filter in an oven at 105°C for 48 hours.

3.2.d. Results

Table 1

	MUCL39210		MUCL28817	
	U/ml	U/g biomass	U/ml	U/g biomass
WT	1,33	92,5	0,45	27,5
Tf. 1	5,75	428	20,18	1127,5
Tf. 2	44,5	3888	1,225	75
Tf. 3	18,5	1488	26,03	1355
Tf. 4	11,9	933	21,53	1145
Tf. 5	20,8	1675	25,13	1325
Tf. 6	21,5	1683	5,925	345
Tf. 7	32,5	2348	24,53	1270
Tf. 8	34,3	2718	7,95	405
Tf. 9	59,5	4035	5,55	307,5
Tf. 10	32,5	2850	22,05	1122,5
Tf. 11	18,4	1430	2,3	142,5
Tf. 12	46	3680	9,85	497,5
Tf. 13			20,3	1127,5
Tf. 14			25,35	1302,5
Tf. 15			2,025	110
Tf. 16			2,1	122,5
Tf. 17			5,35	302,5
Tf. 18			21,63	1205
Tf. 19			2,725	152,5
Tf. 20			25,35	1282,5
Tf. 21			20,38	1032,5
Tf. 22			22,18	1027,5
Tf. 23			1,95	120
Tf. 24			11,05	577,5
Tf. 25			2,95	177,5
Tf. 26			13,2	667,5
Tf. 27			0,825	52,5
Tf. 28			9,675	540
Tf. 29			28,65	1552,5

Tf. 30			1,775	110
Tf. 31			26,18	1422,5
Tf. 32			2,925	200
Tf. 33			3,9	225
Tf. 34			1,55	90
Tf. 35			1,2	82,5
Tf. 36			2,275	157,5
Tf. 37			9,425	560
Tf. 38			1,075	62,5
Tf. 39			4,275	277,5
Tf. 40			25,05	1370
Tf. 41			8,175	462,5
Tf. 42			1,7	110
Tf. 43			1,075	70
Tf. 44			1,15	70
Tf. 45			2,275	147,5
Tf. 46			1,525	105
Tf. 47			1,475	90
Tf. 48			27,75	1420
Tf. 49			0,75	47,5
Average	28,8	2263	10,48	562,5

[0150] The table 1 shows that all transformants (Tf.) obtained with the strain *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 produce significantly more α-amylase than a non-transformed control strain. Many of the *Aspergillus niger* MUCL28817 transformants produce very few α-amylase compared to the non-transformed strain indicating that in those cases, they should not have been transformed by the reported gene or that this reporter gene is integrated under a non-expressible form.

[0151] The *Myrothecium* transformants produce more α-amylase than the *Aspergillus* transformants obtained and cultivated in the same conditions. The best *Xepiculopsis* transformant (n°9) produces more than twice α-amylase compared to the best *Aspergillus* transformant (n°29). On an

average the *Myrothecium* transformants produce 2.75 more α-amylase than the *Aspergillus* transformants.

[0152] Furthermore, the *Myrothecium* transformants have a better specific production of α-amylase (amount of enzyme / g of biomass) compared to the *Aspergillus* transformants.

3.4. Stability of the transformants

[0153] A hygromycin-resistant transformant of *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 was stored at -80°C. During the storage, the same transformant was successively replicated 20 times on solid PDA (Potato Dextrose Agar) medium without selective pressure. After these replica platings, the two strains (the original transformant and the replicated strain) together with the non-transformed *Myrothecium gramineum* (syn. *Xepiculopsis graminae*) MUCL39210 were grown (24°C - 145 rpm) in 20 ml of liquid medium (AMM medium supplemented with 3% sucrose and 0.5% yeast extract, pH 6.3) in 100 ml erlenmeyers. Samples of the cultures were taken at various times and assayed for α-amylase activity.

Table 2

	Activity U/ml	
	120 HF	144 HF
Wild-type	3,225	9,4
Original transformant	15,9	19,575
Transformant replicated 20 times	16,925	21,375

[0154] As shown in the table 2, the number of replications performed with the *Myrothecium gramineum* (syn. *Xepiculopsis graminea*) MUCL39210 transformant does not affect the productivity of the strain.

3.5. Analysis of transformants of other Myrothecium strains

[0155] Similar results (enzymatic activity determination, control of the integration of the DNA constructs) as the ones obtained for transformants of *Myrothecium gramineum* (*Xepiculopsis graminea*) MUCL39210 have been obtained with transformants of other representative strains of the species *Myrothecium gramineum* as well as transformants of strains representative of the genus *Myrothecium*, i.e. *Myrothecium gramineum* IMI140595, *Myrothecium gramineum* CBS449.71, and *Myrothecium verrucaria* CBS328.52. According to the ribosomal DNA sequencing results, it appears that *Myrothecium gramineum* CBS449.71 could belong to another species than *gramineum*.

[0156] The same types of DNA constructs as for *Myrothecium gramineum* (*Xepiculopsis graminea*) MUCL39210 have been used for the transformation of these strains. The transformants obtained have been evaluated as described in example 3.2.

[0157] The strains CBS449.71, IMI140595 and CBS328.52, and their transformants were grown (30°C - 145 rpm) in 70 ml of liquid medium (AMM medium supplemented with 3% sucrose and 0.5% yeast extract, pH 6.3) in 250 ml Erlenmeyer.

[0158] The amylase activity of the strains was measured after 123 hours fermentation. The results of amylase production are presented in table 3.

Table 3

strain		Relative amylase activity
CBS449.71	wt.	1
CBS449.71	transformant n°13	3.44
CBS449.71	transformant n°2	4.23
CBS449.71	transformant n°45	4.23
CBS449.71	transformant n°1	4.97
IMI140595	wt	1
IMI140595	transformant n°22.1	1.86
IMI140595	transformant n°34.1	2.03
IMI140595	transformant n°1.1	13.81
CBS328.52	wt	1
CBS328.52	transformant n°13	1.47
CBS328.52	transformant n°31	5.81
CBS328.52	transformant n°23	28.69

[0159] The applicant has made a deposit of some *Myrothecium* strains according to the invention at the BCCM/MUCL Culture Collection (Mycothèque de l'Université Catholique de Louvain, Place Croix du Sud 3, B-1348 LOUVAIN-LA-NEUVE, BELGIUM). Accession numbers are the following: MUCL44828 for the strain *Myrothecium gramineum* CBS449.71, MUCL44829 for the strain *Myrothecium gramineum* IMI140595, MUCL44830 for the strain *Xepiculopsis graminea* (*Myrothecium gramineum*) MUCL39210, MUCL44831 for the strain *Myrothecium verrucaria* CBS328.52 according to the invention. The above deposits under the Budapest Treaty according to Rule 28 EPC were made on 27 august 2003.